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Associations of Urinary Cadmium with Age and Urinary Proteins: Further Evidence of Physiological Variations Unrelated to Metal Accumulation and Toxicity

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Running title: Significance of low-level urinary cadmium

Key words: body burden, cadmium, retinol-binding protein, tubular dysfunction

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Abbreviations:

BMI: body mass index

Cd-MT: cadmium-metallothionein

GFR: glomerular filtration rate

CI: confidence interval

K-Cd: cadmium in kidney cortex

LMW: low-molecular-weight

U-Alb: albumin in urine

U-Cd: cadmium in urine

U-Creat: creatinine in urine

U-RBP: retinol-binding protein in urine

Abstract

Background: The current risk assessment for environmental Cd largely relies on the assumption that urinary Cd (U-Cd) is a reliable biomarker of the Cd body burden. Recent studies increasingly question the validity of this assumption.

Objectives: We studied the lifetime trend of U-Cd as a function of diuresis, gender, smoking status and protein tubular reabsorption. We also analyzed the associations between U-Cd and urinary proteins.

Methods: Cd, retinol-binding protein and albumin were measured in the urine of six cohorts of the general population of Belgium with a mean age ranging from 5.7 to 88.1 years (N=1,567). Variations of U-Cd with age were modeled by using natural cubic splines.

Results: In both genders, U-Cd in μg/L decreased to a minimum (around 0.20) at the end of adolescence, then increased till the age 60-70 (around 0.60 in never-smokers) before leveling off or decreasing. Expression in μg/g creatinine amplified these variations (minimum, 0.15; maximum, 0.70) and also caused much higher U-Cd values in women. There was no difference in U-Cd levels between never- and former smokers and the difference with current smokers did not increase over lifetime. Lifetime curves of U-Cd were shifted to higher values with increasing urinary retinol-binding protein or albumin, a consequence of the co-excretion of Cd with proteins.

Conclusions: At low exposure levels, U-Cd and age are associated through nonlinear and non-monotonous relationships that appear to be mainly driven by the recent Cd intake and physiological variations in the excretion of creatinine and proteins.

Introduction

Cadmium (Cd), a by-product of zinc production, is one of the most cumulative and toxic metals to which humans can be exposed. The two major sources of Cd exposure for the general population are diet and tobacco smoking. Once absorbed by ingestion or inhalation, Cd efficiently accumulates in the organism, in particular in the kidneys where it is retained with a biological half-life exceeding 20 years. As a consequence, the Cd body burden, which is negligible at birth, rises continuously during life until the age of 60-70 from which it levels off and even decreases. The current consensus is that in chronic Cd intoxication the kidney, the main site of storage, is also the critical target organ i.e. the first organ to be affected. The earliest adverse effect of Cd is an impairment of the tubular function resulting in an increased urinary excretion of low-molecular-weight (LMW) proteins such as retinol-binding protein. This tubular dysfunction develops in a dose-dependent manner, the risk of LMW proteinuria appearing only when the Cd concentration in kidney cortex (K-Cd) reaches a threshold of 150-200 μg/g wet weight (Nordberg et al. 2007).

From a toxicological point of view, Cd presents a rather unique property in that the amount of Cd stored in the kidneys can be estimated non-invasively by measuring the Cd concentration in urine Cd (U-Cd). Studies in industrial workers have shown that before renal dysfunction occurs there is a significant correlation between U-Cd and K-Cd (Roels et al. 1981; 1983). This finding as established in industrial workers was rapidly extended to the general population, an extrapolation based on the assumption that the relationship between U-Cd and the K-Cd holds regardless the route, level and duration of exposure. To our knowledge, only two studies have checked the validity of this assumption. Nilsson et al. (2000) found no significant association between U-Cd and K-Cd determined by an X-ray fluorescence technique in a group of Swedish farmers. Orlowski et al. (1998) found a significant

association but this was in an autopsy study using urine samples contaminated with Cd from bladder autolysis.

Based on the premise that U-Cd reflects Cd body burden, a number of studies have been conducted over the last years among the general population. These studies have reported a wide range of health effects associated with low-level U-Cd, including renal dysfunction, bone demineralization and fractures, reproductive impairment, neurodevelopment deficits, cancers and even mortality (for review see Nordberg et al. 2007; Jarup and Akesson 2009). Recent studies, however, have reported observations that challenge the long-held view that U-Cd is a reliable biomarker of the Cd body burden at low exposure levels. Basically these studies show that U-Cd is strongly influenced by a series of factors unlikely to be related to Cd toxicity or accumulation. Weaver et al. (2011), for instance, have demonstrated a positive correlation between U-Cd and the glomerular filtration rate (GFR), which most probably is the reflection of a faster elimination of Cd with more efficient renal function. Another factor contributing to U-Cd variations is the residual influence of diuresis that persists after correction for urinary creatinine (U-Creat) or for specific gravity (Chaumont et al. 2011, 2012; Haddam et al. 2011). These studies have also provided evidence of a co-excretion of Cd and proteins in urine as a result of the normal physiological variations in the tubular reabsorption of LMW proteins, including Cd-metallothionein (Cd-MT) (Akerstrom et al. 2012; Chaumont et al. 2012; Haddam et al. 2011). To further complicate the picture, it has been suggested that tobacco smoking, long regarded as a mere additional source of Cd, can cause changes in renal function distorting the relationships between the U-Cd and LMW proteinuria (Chaumont et al. 2011).

The objective of the present study was to re-assess the significance of U-Cd as a biomarker of Cd body burden or cumulative exposure to the metal. This has been done indirectly by

comparing the lifetime trend of U-Cd as a function of gender, smoking status, diuresis and urinary protein excretion. We also analyzed the associations between U-Cd and urinary proteins in different age groups, comparing them with the relationship described in an early Cadmibel study (Buchet et al. 1990).

Materials and Methods

Study populations

To perform this study, we used sets of data from six separate epidemiological studies conducted on the general population of Belgium. Three studies were performed in schools with the objective of evaluating the impact of environmental stressors on child or adolescent health. The first study was performed in 2006-2007 among a total of 847 adolescents from three secondary schools (mean age, 15.4 years) (Bernard et al. 2008, Chaumont et al. 2012), the second in 2007-2008 among a total of 430 children in third kindergarten in 30 schools (mean age, 5.7 years) (Voisin et al. 2012), and the third in 2009-2010 on 400 first primary grade children in the same schools as above (mean age, 7.6 years) (Voisin et al. 2013). The three other studies were performed on adults (age range, 18-98 years) living in the same areas. The first study was the Cadmibel study that was carried out in 1985-1989 among 1,220 subjects to assess the health impact of environmental pollution by Cd (Buchet et al. 1990). The second study was conducted in 2001 among 272 subjects with the aim of evaluating dioxin and heavy metal exposure in the vicinity of waste treatment facilities and iron and steel plants (Fierens et al. 2003). The third study in adults was performed in 2011 among 34 nursing home residents. We excluded subjects suffering from diabetes or from chronic disease likely to impact on renal function (n=276) and those who had been occupationally exposed to heavy metals including Cd (n=167). In order to minimize confounding by diuresis, we also excluded subjects with U-Creat < 0.3 and > 3 g/L (n=127). For children in

the third kindergarten and for adolescents, we then randomly selected 200 subjects of both genders. For children in the first primary school, only 95 subjects of both genders could be selected among those who were not retained when they were in third kindergarten. Two individuals in the group of children were excluded for U-RBP as they appeared as outliers in the graph of residuals (102 and 667 µg/L). We also excluded six individuals from the adult nonsmoking group because their U-Cd concentration deviates more than 3 times the SD from the mean calculated in this group. The level of statistical significance was set at $p<0.05$ two sided. Thus the total population (N=1,567) covered all age groups between 4.8 to 98.0 years.

Study protocol

The protocol of all these studies was approved by the Ethics Committee of the Faculty of Medicine of Catholic University of Louvain. Children and adolescents were examined only with the written consent of their parents. Study participants or, for children and adolescents, their parents filled out a self-administered questionnaire about age, gender and factors likely to affect exposure to Cd and the renal function. We measured body weight and height and collected untimed urine samples. All subjects were examined between 9:00 and 16:00. The protocol of the Cadmibel study was slightly different in that subjects provided 24-hour urine sample. Cd was measured in urine by means of inductively coupled argon plasma mass spectrometry (ICP-MS) with an Agilent 7500 instrument (Agilent Technologies. Santa Clara, California, USA). This method was used to determine Cd in all urine samples (children, adolescents, adults and elderly) at the exception of samples from the Cadmibel study. To ensure the comparability of data, we re-analyzed the urine samples of adolescents that had been previously analyzed by the University of Lund in the framework of the Phime project supported by the European Union (Chaumont et al. 2012). Briefly, urine specimens (500 µl) were diluted quantitatively (1+9) with a HNO_3 1%, HCl 0.5% solution containing Sc, Ge, Rh and Ir as internal standards. The detection and quantification limits were 0.02 and 0.05 µg/l,

respectively. The determination of U-Cd in the Cadmibel study was performed by electrothermal atomic absorption spectrometry (Perkin-Elmer Zeeman-3030 or Zeeman-5100) using the stabilized-temperature-platform-furnace techniques coupled with a Zeeman-effect background correction system (see Supplemental Material for the analytical performances of these methods). U-Creat was determined by a modified Jaffé reaction using a Beckman Synchron LX 20 analyzer (Beckman Coulter GmbH, Krefeld, Germany). The concentrations of retinol-binding protein (U-RBP) and albumin in urine (U-Alb) were determined by latex immunoassay (Bernard and Lauwerys 1983). U-Creat in the Cadmibel study was determined on a COBASBIO centrifugal analyzer (Roche Diagnostics).

Statistical analyses

All biological parameters were reported as median and interquartile range and were log-transformed to approximate normal distribution. Student's t-test was carried out to examine gender differences with regard to U-Cd and other urinary parameters. The urinary excretion of these biomarkers was also compared across age groups and according to smoking status by one-way ANOVA followed by the Tukey-Kramer post-hoc test. Variations of U-Cd and other urinary proteins with age were modeled by using natural cubic spline function. Knots were placed at fixed quantiles of the predictor's marginal distribution as suggested by Harrell (2001). Selection of the number and location of the knots were based on minimizing Akaike's Information Criteria. We used the function "ns" in R with the "splines" package to model the natural cubic spline (R 2.14.2, 2012). For these analyses we expressed urinary biomarkers as µg per liter and as µg per g of creatinine. These models were run by stratifying the population according to gender, smoking status and the renal handling of proteins as reflected by tertiles of U-RBP or U-Alb. The latter analysis was done by adjusting U-Cd, U-RBP and U-Alb for the residual influence of diuresis on the basis of the simple linear regression coefficient between U-Creat and these biomarkers.

Associations of U-Cd with biomarkers of renal function were assessed by multiple linear regressions. Stepwise forward selection procedure was applied to select covariables with significance levels of 0.05 for a variable to enter and 0.10 to stay in the model. The tested independent variables were body mass index (BMI), age and gender. We performed these analyses by testing two different models to adjust for the residual effect of diuresis. In a first model, proteins and U-Cd were expressed per g of creatinine and in the second, we expressed the urinary concentrations of these biomarkers per liter. In both models, U-Creat was considered as a separate independent variable to adjust for the residual influence of diuresis as suggested by Barr et al. (2005). The underlying statistical assumptions about the homoscedasticity and normality of the errors were verified visually with regression residuals. Independence of the residuals was assessed by the Durbin-Watson test.. Data were analyzed using R software.

Results

Characteristics of the studied populations and the concentrations of U-Cd and other urinary biomarkers are summarized in Table 1 for children and adolescents. Tables 2 and 3 show the same variables for adults and elderly divided according to smoking status and gender, respectively. Mean age of studied populations ranged from 5.7 to 88.1 years with an overall range extending from 4.8 to 98 years. When considering all groups except smokers, the median U-Cd levels ranged from 0.24 in children to 0.62 in elderly when expressed in µg/L, and from 0.16 in adolescents to 0.60 in elderly when expressed in µg/g creatinine. In adults, the median U-Cd expressed per liter or g of creatinine was higher in women than in men (never-smoker subjects, 0.46 *vs* 0.36 µg/L and 0.57 *vs* 0.27 µg/g creatinine) and in current smokers compared to former (0.64 *vs* 0.52 µg/L and 0.67 *vs* 0.48 µg/g creatinine) or never-smokers (0.42 µg/L and 0.43 µg/g creatinine). By contrast, there were no differences in the

U-Cd between males and females of other groups, nor between former and never-smokers among adults. In all age groups except the elderly, U-Cd expressed in µg per g of creatinine was negatively correlated to U-Creat (children, n=296, r^2=0.171, p<0.001; adolescents, n=200, r^2=0.073, p<0.001; adults, n=1,048, r^2=0.108, p<0.001; elderly, n=23, r^2=0.140, p=0.08) indicating a residual influence of diuresis due to an over-adjustment on the basis of U-Creat. The number of cigarettes smoked per day and the number of pack-years (only available for 358 current smokers: median, 20 and 18, respectively) were positively correlated with U-Cd (n=358, r^2=0.033, p<0.001 and r^2=0.30, p<0.001, respectively) and U-Alb (n=330, r^2=0.025, p=0.004 and r^2=0.033, p=0.002, respectively) but not with U-RBP (n=330, r^2=0.0003, p=0.92 and r^2=0.003, p=0.35) (urinary parameters expressed per g of creatinine).

Even though all analyses were performed in the same laboratory, we ascertained the comparability of the Cadmibel data with values observed in the same areas more than 15 years later. We checked the comparability of these data by analyzing the relationships linking U-Cd to age. As shown in Supplemental Material, Figure S1, these relationships were superposed to each other with virtually identical slopes and intercepts. Accordingly, the mean concentrations of U-Cd adjusted for the age of 50 did not differ between the two studies (0.48 for Cadmibel population *vs* 0.44 µg/g creatinine for the population recruited in 2001, p>0.05).

Variations of U-Cd and proteins over lifetime were derived from linear regression including age as a natural cubic spline function and by combining all data except those from smokers. These models were developed separately according to gender and according to whether urinary parameters were expressed per liter or per g of creatinine. As shown in Figure 1 A,B, whatever the tested model, relationships of U-Cd with age are nonlinear and non-monotonous over lifetime. When expressed as µg per liter (Figure 1A), an increase of U-Cd with age is

seen in both genders starting from the age of 25 (U-Cd, 0.23 µg/L) till the age of 60-70 (0.64 µg/L), when a decrease is observed in women and a leveling off appears in men. Over that period, U-Cd was increased by a factor of about three in both genders. Before adulthood, there were by contrast little variations of U-Cd, or if anything, a small peak in children around the age of 8 years. Basically, the same patterns of change with age were seen when U-Cd was expressed per g of creatinine (Figure 1B), with however an overt amplification of the variations before adulthood as well as between genders. In particular, a two-fold decrease of U-Cd was observed in adolescents compared to children and from then, U-Cd became systematically lower in men than in women. When expressed per g of creatinine, the rise of U-Cd during adulthood was also amplified with an average four-fold increase of U-Cd between the age of 20 and of 60-70 (U-Cd, 0.16 to 0.72 µg/g creatinine).

Supplemental Material, Figure S2 displays the fitted curves of U-Creat and proteins changes over lifetime and according to gender. Clearly in both genders, U-Creat increases in chidlhood to reach a maximum value at the adolescence and then steadily decreases with age but much more abruptly in women, which creates a systematic gender difference over the rest of life. This gender-related variation is just the opposite of that observed with U-Cd per g of creatinine. Like for U-Creat, the excretion of U-RBP and U-Alb showed a sharp transient increase in the adolescence followed by a progressive increase with aging. While these two phases are present for U-RBP expressed per liter and per g creatinine, they seem however to be preceded by a sharp decline in U-RBP excretion occurring during early childhood.

To gain further insight into the relationships between U-Cd and the cumulative exposure to Cd, we compared the evolution of U-Cd with age according to smoking status (Figure 2 A,B). Until the age of 70, U-Cd levels were systematically higher in current smokers than in never smokers. This difference however did not really increase over lifetime contrary to the

intuitive expectation for a biomarker supposed to reflect the increase of the Cd body burden with chronic smoking. Indeed, at the age of 20, 40 and 60 years, mean U-Cd concentrations expressed per liter (or per g of creatinine) of current smokers exceeded that of never smokers by 45% (50%), 70% (76%) and 47% (49%), respectively. The pattern of U-Cd with age in former smokers also did not show the expected difference with never-smokers, the two groups reaching practically the same mean U-Cd level at the age of 60. If anything, former smokers showed even a lower mean U-Cd concentration after the age of 60.

We completed our analyses by evaluating the influence of the renal handling of proteins on the lifetime patterns of U-Cd. First, we applied multiple linear regression analyses to assess the associations between U-Cd and U-RBP or U-Alb expressed per liter or per g creatinine, in the different age groups. Whatever the model tested there was no association between U-Cd and U-Alb. By contrast, U-RBP was consistently associated with U-Cd and BMI among children, adolescents and adults regardless the smoking status (Table 4). Paradoxically, associations between U-Cd and U-RBP were the strongest in children and especially in adolescents, i.e. the age groups with the lowest median U-Cd levels. Second, we pursued these analyses by comparing the lifetime patterns of U-Cd in the whole population stratified according to tertiles of U-RBP. The same analysis was also done with albumin as recent studies provide evidence of a co-excretion between Cd and albumin (Akerstrom et al. 2012; Chaumont et al. 2012). Variations in Cd intake were minimized by excluding former and current smokers. We also minimized the variations due to diuresis by adjusting both U-Cd and urinary proteins for the residual associations with U-Creat observed in each age group. Figure 3 A,B shows that in adults and in adolescents there is a shift of U-Cd to higher values with increasing U-RBP and U-Alb. At the age of 60, U-Cd reached value that was approximately 30% higher in the highest tertile of U-RBP compared to the intermediate or lowest tertile (Figure 3A). U-Cd levels were similarly increased by the higher U-Alb

excretion, with about a 20% difference between the highest and the lowest tertiles of U-Alb from puberty till the age 60 (Figure 3B).

Discussion

The present study has been conducted on six groups of the general population of different age living in Brussels and in the southern part of Belgium. The U-Cd levels in our study are very similar to those reported in Canada (Health Canada 2010) and in several European countries (Aguilera et al. 2010; Dhooge et al. 2010; Pennemans et al. 2011). These U-Cd concentrations were, however, about two or three times higher than U-Cd observed in the United States (CDC 2009) or Germany (Wilhelm et al. 2005). Our findings clearly show that at these low levels of exposure U-Cd increases with age in a non-monotonous manner. The evolution of U-Cd over lifetime is indeed characterized by a transient increase during childhood followed by a decrease during adolescence and then a progressive rise till the age of 60, from which U-Cd levels off or even decreases. These variations are amplified by the creatinine adjustment as a result of age-related variations in U-Creat, which in both genders peaks at puberty and then progressively declines over the rest of life. In adults, U-Cd curves were systematically shifted to higher values when comparing women to men and current to never-smokers. By contrast, U-Cd did not vary significantly with gender in children and adolescents, or with past smoking in adults. In addition, U-Cd was consistently associated with U-RBP in all age groups with the exception of elderly.

The patterns of U-Cd variations over lifetime observed in our study are in discordance with the mathematical models predicting a monotonous curvilinear increase of U-Cd with age until a leveling off occurs around 60-70 (Amzal et al. 2009). Our findings are also not consistent with data from autopsy studies or from models describing an almost linear increase of K-Cd from birth until 50-60 where a plateau or a decrease is seen (Benedetti et al.1999).

Interestingly, models taking into account the variations in kidney weight with age predict a small decrease of both K-Cd and U-Cd during puberty, a phenomenon similar to that observed with U-Cd in our study (Friberg et al. 1974; Ruiz et al. 2010). Clearly, values of U-Cd observed during childhood or adolescence cannot be interpreted as a reflection of K-Cd. Children aged 8 years have indeed about the same U-Cd levels as never-smokers aged 40 years. The patterns of U-Cd in our study really match that of K-Cd only between the ages of 20-60. This matching holds even in quantitative terms as the relative increases of U-Cd during that period (3-fold for U-Cd per liter and 4-fold for U-Cd per g of creatinine) are for both genders in the same range as that observed with K-Cd in the nonsmoking general population (Benedetti et al. 1999). However, caution should be exercised before interpreting this similarity as the evidence that U-Cd reflects a concomitant increase of K-Cd. Unexpectedly indeed, the pattern of U-Cd increase with age did not differ between former and never smokers. In addition, the difference of U-Cd between current and never-smokers remained fairly constant over lifetime, instead of increasing as described for K-Cd (Hahn et al. 1987). These results are in agreement with a previous study in which no significant difference in U-Cd was observed between former and never smokers (Ikeda et al. 2005). These results suggest that the higher U-Cd observed among current smokers mainly reflects the higher intake of Cd from tobacco smoke rather than the increasing Cd renal burden with chronic smoking.

The explanation for these discordant patterns of U-Cd and K-Cd with age has probably to be sought for in the two basic mechanisms that govern the urinary excretion of Cd and whose respective contribution varies with the level of Cd exposure and the integrity of the renal function. The first mechanism is the release or secretion of Cd accumulated in the kidney. This mechanism is probably responsible for the parallel rise of U-Cd with the Cd renal burden described in industrial workers and experimental animals with a preserved renal

function (Nordberg et al. 2007). This mechanism explains why in workers with no tubular dysfunction U-Cd remains very stable and elevated many years after removal from exposure (Friberg 1984). The second mechanism is the glomerular filtration of Cd-MT followed by the excretion of Cd-MT unreabsorbed by the proximal tubule. In this mechanism, the urinary output of Cd is determined both by the amount of circulating Cd - thus by the Cd intake - as well as by the capacity of the kidneys to filter and reabsorb LMW proteins. Our data suggest that this second mechanism is predominant in determining U-Cd when the Cd body burden is very low. In that case, indeed, there is a minimal release of Cd from kidneys into urine and also a minimal contribution of the body burden to circulating Cd. This situation explains why children have U-Cd levels similar to that of middle-aged adults, their Cd intake being indeed higher than that of adults (EFSA 2012). This second mechanism seems also to operate during adulthood as evidenced by the increased U-Cd with increasing U-RBP or U-Alb, a consequence presumably of the co-excretion mechanisms (Akerstrom et al. 2012; Chaumont et al. 2012; Haddam et al. 2011).

The only period of life when U-Cd might reflect K-Cd is between puberty and the age of 60-70 when U-Cd raises with age in a similar way as K-Cd regardless the physiological variations in creatinine and protein excretion. But even at that stage, the significance of U-Cd on an individual basis remains uncertain and might be equivocal. The patterns of U-Cd increase with age in current and former smokers are indeed not consistent with that described for K-Cd in autopsy studies. These findings added to the evidence of a co-excretion mechanism of U-Cd with urinary proteins, suggest that even in adults, U-Cd might be more a reflection of the current Cd intake and of the protein reabsorption capacity of the kidney. Actually, the latter determinant might lead to an inverse relationship between U-Cd and K-Cd as, for a similar intake, less tubular reabsorption of the metal means less accumulation in the kidney. This situation is reminiscent of the decline of the K-Cd in subjects who have

developed kidney damage as a result of high industrial or environmental exposure to the metal (Nordberg et al. 2007). Likewise, the decrease of U-Cd observed in the elderly might mirror a parallel decline of the Cd renal burden, even though other aging-related explanations are possible such as a reduced Cd intake or a lower glomerular filtration of Cd-MT.

Our study presents several limitations. Because of ethical and practical reasons it was not possible to collect blood and timed urine samples in all studied groups. Therefore, we could not measure blood Cd, a biomarker of current Cd exposure. A comparison of the variations of urinary and blood Cd over lifetime would have been particularly interesting to determine the relative sensitivities of these two biomarkers to physiological changes occurring during development and ageing. We could not either calculate the creatinine clearance to estimate the GFR, a factor influencing positively the renal elimination of Cd (Weaver et al. 2011).

In conclusion, our study clearly shows that the curvilinear relationship between U-Cd and K-Cd described in industrial workers and assumed in recent models does not hold for the whole general population with a low-environmental exposure to Cd. Over lifetime, U-Cd shows age-related variations that appear to be largely determined by the recent Cd intake and by the renal handling of proteins, and in particular of LMW proteins. These findings are particularly relevant for epidemiological studies of health risks associated with low environmental exposures to Cd. Observations in these studies based on U-Cd would be substantiated by the use of cumulative intake indicators that are unlikely to be confounded by recent Cd exposure and physiological variations in the renal elimination of the metal. Various indicators might be used for that purpose based, for instance, on the residence time in the studied area, on the consumption of locally produced foods or even better on the Cd dietary intake estimated from food contamination data.

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Table 1: Characteristics and biological parameters of children and adolescents

	School children				Adolescents	
	2007-2008		2010		2006	
	Boys	Girls	Boys	Girls	Boys	Girls
N	107	92	49	48	101	99
Age, years	5.6 (4.8-6.8)	5.7 (4.9-6.3)	7.6 (6.0-8.9)	7.7 (6.7-8.8)	15.6 (14.0-17.8)	15.6 (14.2-17.3)
BMI, kg/m^2	20.7±3.0	20.4±3.5	17.4±2.2	17.2±2.8	20.7±3.2	20.6 ± 2.6
Creatinine (g/L)	0.74 (0.58-0.93)	0.66 (0.51-0.90)	0.95 (0.83-1.27)	0.92 (0.63-1.11)	1.65 (1.16-1.96)	1.65 (1.30-2.0)
Cadmium						
µg/L	0.24 (0.17-0.30)	0.23 (0.18-0.32)	0.29 (0.24-0.37)	0.23 (0.18-0.37)	0.24 (0.19-0.32)	0.27 (0.20-0.34)
µg/g creatinine	0.32 (0.25-0.45)	0.36 (0.28-0.47)	0.31 (0.25-0.36)	0.32 (0.25-0.36)	0.16 (0.13-0.20)	0.16 (0.13-0.22)
Retinol-binding protein						
µg/L	97 (70.0-157)	110 (78.0-140)	86.0 (63.0-119)	80.0 (42.0-123)	158 (100-269)	173 (107-265)
µg/g creatinine	143 (103-206)	163 (131-207)	88.0 (65.5-114)	89.6 (60.3-127)	113 (74.4-152)	113 (80.9-150)
Albumin						
mg/L	1.80 (1.00-3.88)	2.70 (1.30-5.55)	3.50 (2.40-6.25)	5.85 (2.80-9.40)	5.72 (2.28-17.9)	9.68 (4.73-42.9)
m/g creatinine	2.62 (1.45-4.71)	4.09 (2.27-7.48)	3.52 (2.28-5.96)	6.77 (3.02-11.3)	3.80 (1.83-11.0)	6.21 (3.42-26.3)

Values are mean (min-max) for age, mean±SD for BMI and median (interquartile range) for urinary biomarkers

Table 2: Characteristics and biological parameters of adult population according to smoking status

	Adults-general population						Elderly-nursing homes		
	1985-1989			2001-2002			2011		
	Never smokers	Former smokers	Current smokers	Never smokers	Former smokers	Current smokers	Never smokers	Former smokers	Current smokers
N	331	155	333	146	52	31	13	6	4
Men, n (%)	88 (26.6)	90 (58.1)	158 (47.4)	59 (40.4)	38 (73.1)	18 (58.1)	1 (7.7)	4 (66.7)	1 (25.0)
Age, years	49.8 (19-88)	50.3 (20-85)	43.6 (18-80)	51.4 (25-71)	51.3 (33-80)	50.4 (33-69)	88.0 (79-98)	76.7 (70-88)	76.8 (61-83)
BMI, kg/m^2	25.1±4.4	25.9±4.4	24.1±4.2	26.3±4.9	27.2±3.5	25.9±4.3	22.4±2.2	23.3±6.3	21.3±1.0
Creatinine (g/L)	0.84 (0.59-1.23)	0.92 (0.69-1.22)	0.94 (0.62-1.45)	1.34 (0.96-1.70)	1.32 (1.05-1.90)	1.37 (1.01-2.07)	0.94 (0.62-1.67)	1.08 (0.77-1.24)	1.13 (0.59-1.74)
Cadmium									
µg/L	0.40 (0.25-0.67)	0.49 (0.32-0.72)	0.62 (0.38-1.04)	0.56 (0.35-0.93)	0.68 (0.42-0.98)	1.12 (0.67-1.82)	0.62 (0.50-1.03)	0.82 (0.34-1.09)	0.91 (0.45-2.15)
µg/g creatinine	0.49 (0.27-0.85)	0.52 (0.32-0.82)	0.67 (0.38-1.15)	0.43 (0.28-0.75)	0.45 (0.33-0.70)	0.68 (0.51-1.19)	0.60 (0.51-0.87)	0.56 (0.41-1.52)	0.95 (0.48-2.50)
Retinol-binding protein									
µg/L	76.0 (48.0-130)	79.0 (50.0-130)	90.0 (55.8-155)	/	/	/	108 (72.3-182)	107 (61.0-276)	85.5 (45.5-223)
µg/g creatinine	91.0 (60.9-139)	81.7 (59.1-116)	97.1 (67.2-140)	/	/	/	96.2 (71.3-235)	100 (76.9-134)	71.7 (43.1-167)
Albumin									
mg/L	8.12 (4.68-14.8)	6.38 (4.13-12.4)	7.52 (4.21-13.9)	/	/	/	13.0 (5.75-42.3)	21.0 (13.0-170)	43.5 (4.50-143)
mg/g creatinine	10.3 (4.06-24.2)	7.20 (3.75-18.1)	9.14 (3.28-19.6)	/	/	/	19.7 (6.58-52.9)	23.8 (12.3-85.1)	33.1 (6.44-156)

Values are mean (min-max) for age, mean±SD for BMI and median (interquartile range) for urinary biomarkers

Table 3: Characteristics and biological parameters of adult population according to gender

	Adults-general population				Elderly-nursing homes	
	1985-1989		2001-2002		2011	
	Men	Women	Men	Women	Men	Women
N	336	483	115	114	6	17
Age, years	46.9 (19-88)	47.7 (18-88)	51.3 (30-80)	51 (25-73)	76.3 (61-88)	85.5 (71-98)
BMI, kg/m^2	25.2±3.5	24.6±4.9	26.3±3.6	26.4±5.4	24.2±5.9	22.3±1.9
Creatinine (g/L)	1.18 (0.81-1.58)	0.75 (0.55-1.10)	1.38 (1.05-1.90)	1.24 (0.93-1.63)	1.45 (1.11-2.01)	0.79 (0.62-1.48)
Cadmium						
µg/L	0.52 (0.32-0.88)	0.47 (0.29-0.79)	0.52 (0.32-0.94)	0.72 (0.46-1.26)	0.96 (0.81-1.09)	0.62 (0.47-1.07)
µg/g creatinine	0.50 (0.28-0.84)	0.62 (0.35-1.05)	0.38 (0.25-0.60)	0.65 (0.40-0.93)	0.66 (0.41-0.66)	0.60 (0.45-1.31)
Retinol-binding protein						
µg/L	108 (61.0-160)	74.0 (46.0-120)	/	/	200 (95.0-387)	79.0 (65.5-142)
µg/g creatinine	91.6 (61.7-133)	91.1 (63.3-139)	/	/	127 (47.2-233)	84.0 (71.3-185)
Albumin						
mg/L	5.20 (2.97-10.1)	9.95 (5.96-16.7)	/	/	26.5 (5.0-270)	13.0 (5.75-52.8)
mg/g creatinine	4.91 (2.0-11.4)	13.4 (5.69-27.6)	/	/	18.7 (7.77-85.0)	19.7 (8.19-53.7)

Values are mean (min-max) for age, mean±SD for BMI and median (interquartile range) for urinary biomarkers

Table 4: Multiple regression analysis of the determinants of urinary excretion of RBP in the general population stratified by age groups and adult smoking habits

		U-RBP (µg/ g creatinine)				U-RBP (µg/L)			
Subjects	N	Independent variable	Coefficient (95% CI)	p	R^2	Independent variable	Coefficient (95% CI)	p	R^2
Children	296	U-Cd	0.217 (0.057, 0.376)	0.007	0.177	U-Cd	0.198 (0.025, 0.375)	0.026	0.300
		Age	-0.100 (-0.127, -0.073)	<0.001		U-Creat	0.756 (0.561, 0.944)	<0.001	
						Age	-0.098 (-0.126, -0.069)	<0.001	
Adolescents	200	U-Cd	0.313 (0.096, 0.529)	0.005	0.093	U-Cd	0.363 (0.139, 0.587)	0.002	0.409
		BMI	-1.07 (-1.66, -0.48)	<0.001		BMI	-1.07 (-1.65, -0.477)	<0.001	
						U-Creat	0.808 (0.549, 1.07)	<0.001	
Adults (19-70 years)									
All	744	U-Cd	0.125 (0.073, 0.177)	<0.001	0.049	U-Cd	0.116 (0.060, 0.173)	<0.001	0.391
		BMI	-0.546 (-0.786, -0.305)	<0.001		BMI	-0.545(-0.785, -0.304)	<0.001	
						U-Creat	0.846 (0.761, 0.932)	<0.001	
Never smokers	284	U-Cd	0.111 (0.029, 0.193)	0.008	0.034	U-Cd	0.103 (0.007, 0.200)	0.035	0.389
		BMI	-0.577 (-0.982, -0.173)	0.005		BMI	-0.580 (-0.986, -0.174)	0.005	
						U-Creat	0.872 (0.733, 1.01)	<0.001	
Ever smokers	460	U-Cd	0.138 (0.068, 0.211)	<0.001	0.052	U-Cd	0.127 (0.052, 0.203)	0.001	0.381
		BMI	-0.515 (-0.978, -0.181)	<0.001		BMI	-0.504 (-0.809, -0.198)		
						U-Creat	0.822 (0.709, 0.935)		
Adults (>70 years)									
All	98	U-Cd	0.013 (-0.391, 0.188)	0.49	0.005	U-Cd	-0.077 (-0.382, 0.227)	0.6	0.197
						U-Creat	1.21 (0.720, 1.69)	<0.001	
Never smokers	62	U-Cd	-0.076 (-0.406 to 0.255)	0.65	0.003	U-Cd	-0.053 (-0.392 to 0.286)	0.8	0.259
						U-Creat	1.22 (0.673 to 1.76)	<0.001	
Ever smokers	36	U-Cd	-0.128 (-0.699 to 0.443)	0.65	0.006	U-Cd	-0.114 (-0.731 to 0.503)	0.7	0.092
						U-Creat	1.19 (0.156 to 2.21)	0.025	

Figure Legends

Figure 1: Associations of U-Cd in µg/L (A) or in µg/g creatinine (B) with age according to gender in healthy and nonsmoking male (n=405) and female (n=581) subjects. Data were fitted using natural cubic splines function with 4 knots placed at the 20th, 40th, 60th and 80th percentiles. Level of statistical difference of U-Cd between male and female was reached from the age of 60 when U-Cd was expressed in µg/L, and from the age of 20 when U-Cd was expressed in µg/g creatinine.

Figure 2: Associations of U-Cd in µg/L (A) or in µg/g creatinine (B) with age according to smoking status. The relationship was modeled using a natural cubic spline function with three knots placed at 25th, 50th and 75th percentiles. In adults aged 18-40, U-Cd of both current and former smokers was significantly higher than in never smokers whereas after the age of 40, there was only a significant difference between current and never smokers. Never smokers, n=490; former smokers, n=213; current smokers, n= 368.

Figure 3: Association between U-Cd and age according to tertiles of U-RBP and of U-Alb in the nonsmoking population (n=840). The relationship was modeled using a natural cubic spline function with three knots placed at 25th, 50th and 75th percentiles. Values of U-Cd were significantly different between tertiles for U-RBP among adolescents only, and for U-Alb from adolescence when combining all subjects.

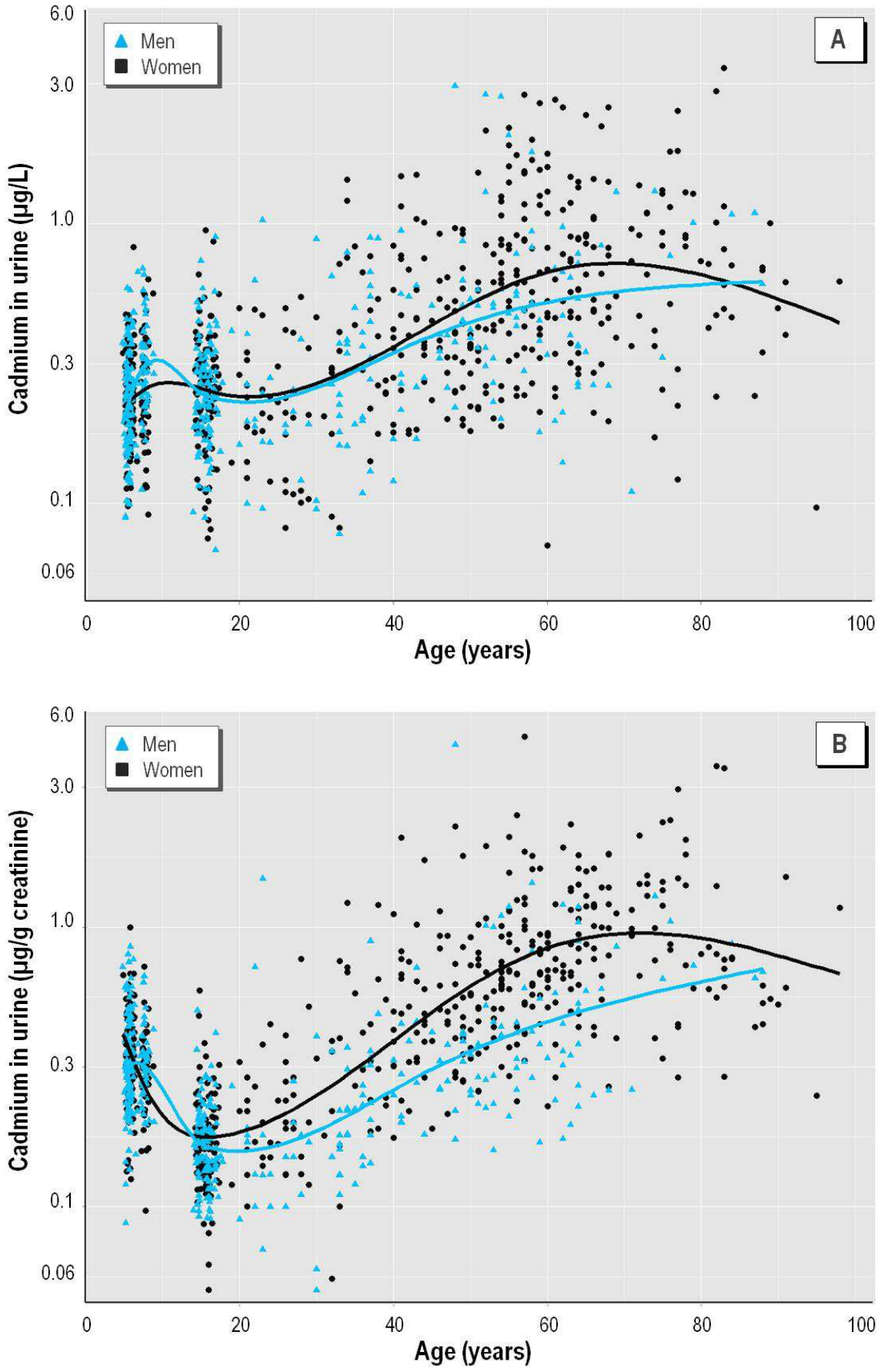
A
Men
Women
Cadmium in urine (µg/L)
6.0
3.0
1.0
0.3
0.1
0.06
0
20
40
60
80
100
Age (years)
B
Men
Women
Cadmium in urine (µg/g creatinine)
6.0
3.0
1.0
0.3
0.1
0.06
0
20
40
60
80
100
Age (years)

Figure 1

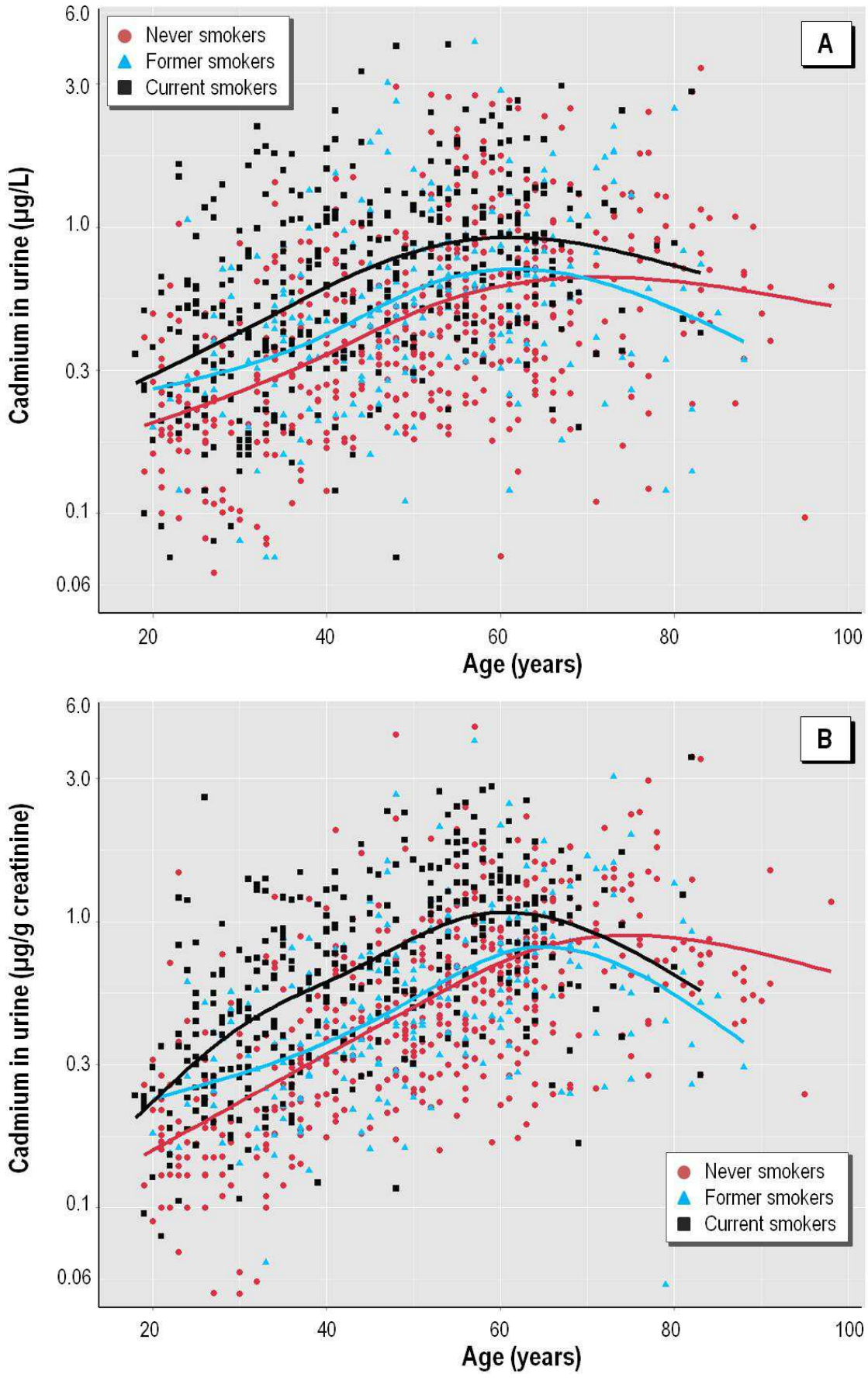
A
Never smokers
Former smokers
Current smokers
Cadmium in urine (µg/L)
6.0
3.0
1.0
0.3
0.1
0.06
20
40
60
80
100
Age (years)
B
Never smokers
Former smokers
Current smokers
Cadmium in urine (µg/g creatinine)
6.0
3.0
1.0
0.3
0.1
0.06
20
40
60
80
100
Age (years)

Figure 2

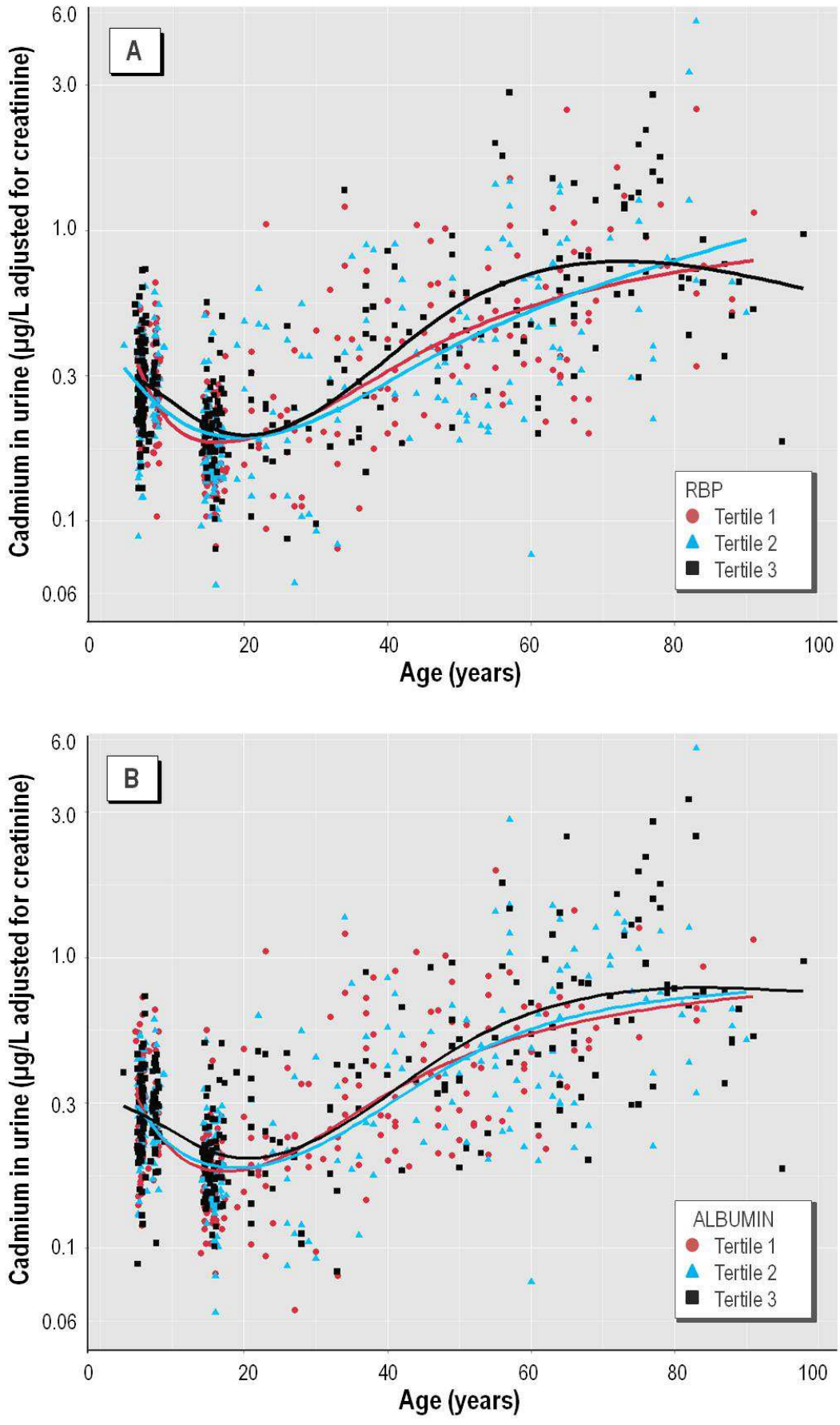
A
B
Cadmium in urine (µg/L adjusted for creatinine)
6.0
3.0
1.0
0.3
0.1
0.06
0
20
40
60
80
100
Age (years)
RBP
Tertile 1
Tertile 2
Tertile 3
ALBUMIN
Tertile 1
Tertile 2
Tertile 3

Figure 3